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Actions of Ethanol on Voltage-Sensitive Sodium Channels: Effects of Acute and Chronic Ethanol Treatment[1]

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ABSTRACT

The effects of acute and chronic ethanol treatment on neurotoxin-stimulated $^{22}Na^+$ uptake and [^{3}H]batrachotoxinin A20-α-benzoate binding to neuronal sodium channels were studied in rat forebrain synaptosomes. Fluorescence measurements were used to assess the intrinsic order or fluidity and the sensitivity to ethanol of rat forebrain synaptic plasma membranes at various intervals during and after chronic ethanol treatment. Acute ethanol administration had no significant effect on neurotoxin binding in the absence or presence of ethanol *in vitro* or on sodium uptake in the absence of ethanol *in vitro*. However, a single dose of ethanol produced a dose and time-dependent attenuation of the inhibitory effect of ethanol on sodium uptake, suggestive of acute tolerance. Chronic ethanol treatment reduced the influx of $^{22}Na^+$ in the presence of batrachotoxin and diminished the inhibitory effect of ethanol *in vitro* on sodium uptake for up to 20 days after withdrawal, but the specific binding of the neurotoxin in the presence or absence of ethanol was unchanged. Synaptic plasma membranes from chronic ethanol-treated rats showed no change in intrinsic order but the disordering effect of ethanol was significantly smaller for up to 20 days after withdrawal. Results of this study demonstrate that brain tissue from ethanol-treated rats can adapt rapidly to some effects of ethanol and that chronic ethanol administration can reduce the effects of ethanol on physical and functional properties of neurons for a prolonged period of time.

Ethanol-induced changes in the physical properties of biological membranes are thought to be involved in the diverse pharmacological actions of ethanol (Goldstein, 1984; Harris and Hitzemann, 1981). Changes in the physical properties of neuronal membranes may induce alterations in the functional properties of nervous tissues (*e.g.* enzymatic activity, ion transport and neurotransmitter regulation) resulting in impaired signal transduction and information processing and, ultimately, the behavioral manifestations of intoxication. At the present time, it is thought that ethanol and related membrane perturbants modulate the fluidity of nerve membranes by disordering the lipid portions of brain membranes (Chin and Goldstein, 1977a; Harris and Schroeder, 1981, 1982; Crews *et al.*, 1983).

Chronic exposure to ethanol has been shown to produce tolerance and physical dependence (Majchrowicz and Hunt, 1976; Ritzmann and Tabakoff, 1976). Furthermore, neuronal membranes derived from ethanol-tolerant animals are resistant to the disordering effect of ethanol *in vitro* (Chin and Goldstein, 1977b; Harris *et al.*, 1984). The mechanisms involved in the observed tolerance to the disordering effect of ethanol *in vitro* are not yet known but adaptive changes in the lipid composition of neuronal membranes have been examined for possible involvement. Membrane cholesterol content has been reported to be increased (Chin *et al.*, 1978; Smith and Gerhart, 1982), decreased (Harris *et al.*, 1984) or unchanged (Johnson *et al.*, 1979; Lyon and Goldstein, 1983) after chronic ethanol treatment. However, the reported change in cholesterol content is rather small in magnitude. Likewise, chronic ethanol treatment has been reported to produce only slight changes in the acyl composition of synaptosomal phospholipids (Sun and Sun, 1979; Alling *et al.*, 1982; Crews *et al.*, 1983; Harris *et al.*, 1984). Recently, it was shown that the membrane ganglioside content and acyl composition of gangliosides were unchanged in ethanol-tolerant mice (Harris *et al.*, 1984). Thus, it appears that chronic ethanol treatment consistently alters membrane physical properties in the absence of any marked changes in lipid composition.

The results of a number of recent studies suggested that some of the properties of voltage-sensitive sodium channels of brain

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ABBREVIATIONS: BTX-B, batrachotoxin A 20-α-benzoate; BTX, batrachotoxin; HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene.

synaptosomes are sensitive to the physical properties of membranes. In a group of chemically diverse membrane perturbants, the magnitude of the inhibition of neurotoxin-stimulated sodium influx was proportional to the degree of lipid disordering in the membrane core (Harris and Bruno, 1985a,b). Furthermore, for a series of aliphatic alcohols, there was an excellent correlation between the potency for inhibition of neurotoxin-stimulated sodium influx and the membrane/buffer partition coefficient, suggesting that a hydrophobic site in the membrane was involved in the action of the alcohols (Mullin and Hunt, 1984, 1985). In the present study we sought to determine whether acute or chronic ethanol treatment altered some of the properties of voltage-sensitive sodium channels in rat brain synaptosomes. We also monitored the physical properties of brain membranes at various intervals during and after chronic ethanol treatment.

Methods

Animals and chemicals. Male Sprague-Dawley rats (200–300 g) were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA) and were housed two per cage with free access to water and standard laboratory chow. Chemicals and suppliers were as follows: scorpion (Leiurus quinquestriatus) venom, tetrodotoxin and veratridine from Sigma Chemical Co. (St. Louis, MO); [benzoyl-2,5-^{3}H]BTX-B (51 Ci/mmol) and carrier-free ^{22}NaCl were from New England Nuclear (Boston, MA) Fluorescent probes were obtained from Molecular Probes, Inc. (Junction City, OR). BTX was kindly supplied by Dr. John Daly (National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD). All other chemicals were obtained from commercial sources and were of analytical grade.

Preparation of synaptosomes. For measurement of [^{3}H]BTX-B binding and BTX-stimulated $^{22}Na^+$ influx a crude synaptosomal (P_2) fraction was prepared by a modification of the method of Gray and Whittaker (1962). Immediately after decapitation the whole brain was removed, the cerebellum and brainstem were discarded and the tissue was homogenized in 0.32 M sucrose and 5 mM K_2HPO_4, pH 7.4 (10 ml/g wet weight), with 10 strokes of a motor driven Teflon-glass homogenizer. The homogenate was then centrifuged at 1000 × *g* for 10 min. The resulting supernatant was then centrifuged at 17,000 × *g* for 60 min. The final pellet was resuspended in ice-cold buffer containing (millimolar): KCl, 5.4; $MgSO_4$, 0.8; glucose, 5.5; HEPES-Tris (pH 7.4), 50; and choline chloride, 130. Ten strokes of a loose fitting glass-glass homogenizer were used to resuspend the final pellet. Synaptosomes were kept on ice and were used immediately after preparation.

Measurement of $^{22}Na^+$ uptake. Neurotoxin-stimulated uptake of $^{22}Na^+$ was determined by a modification of the method of Tamkun and Catterall (1981). Aliquots (50 μl) of the synaptosomal suspension were preincubated at 36°C for 2 min with buffer only or buffer containing the indicated concentration of ethanol. Immediately after the preincubation with ethanol the indicated concentration of BTX was added and the samples were incubated for 10 min at 36°C. After 10 min, the samples were diluted with a solution containing (final concentration, millimolar): KCl, 5.4; $MgSO_4$, 0.8; glucose, 5.5; HEPES-Tris (pH 7.4), 50; choline chloride, 128; NaCl, 2; ouabain, 5; 1.3 μCi of carrier-free ^{22}NaCl per ml and the indicated concentration of ethanol and BTX (micromolar). After a 5 sec incubation, the uptake of $^{22}Na^+$ was terminated by the addition of 3 ml of an ice-cold wash solution containing (millimolar): choline chloride, 163; $MgSO_4$, 0.8; $CaCl_2$, 1.8; HEPES-Tris (pH 7.4), 5; and bovine serum albumin, 1 mg/ml. The mixture was filtered rapidly under vacuum through a 0.45-μm cellulose filter (Amicon, Lexington, MA or Schleicher and Schuell, Keene, NH) and the filters were washed twice with 3 ml of wash solution. Filters were placed in scintillation vials with 15 ml of scintillation cocktail and filter radioactivity was determined by liquid scintillation spectrometry. The data are presented as corrected specific uptake after subtraction of nonspecific uptake (tetrodotoxin, 1 μM present in buffers).

Measurement of [^{3}H]BTX-B binding. The binding of [^{3}H]BTX-B was measured by a modification of the method of Catterall *et al.* (1981) as described in detail in the accompanying manuscript (Mullin and Hunt, 1987).

Fluorescence measurements. A HH-1 T-format polarization spectrofluorimeter (BHL Associates, Burlingame, CA) with fixed excitation and emission polarization filters was used to measure fluorescence intensity parallel and perpendicular to the polarization phase of the exciting light (Harris and Schroeder, 1982). Polarization of fluorescence and intensity of fluorescence were calculated by an on-line microprocessor. Similar instrumentation is presented in more detail by Johnson *et al.* (1979). The fluorescent probes DPH and TMA-DPH were used. The excitation wavelength was 362 nm, a 03FCG001 filter (Melles Griot, Irvine, CA) was used in the excitation beam and KV 389 filters (Schott Optical, Duryea, PA) were used for emission. Cuvette temperature was maintained by a circulating water bath and monitored continuously by a thermocouple inserted into the cuvette to a level just above the light beam.

Synaptic plasma membranes (SPM-2) were used for all fluorescence measurements. The cerebellum and brainstem were removed from the brain and SPM-2 were prepared by Ficoll and sucrose density centrifugation (Fontaine *et al.*, 1980; Harris and Schroeder, 1982). Membranes were resuspended in phosphate-buffered saline containing (millimolar): NaCl, 136; KCl, 2.7; KH_2PO_4, 1.5; $Na_2HPO_4 \cdot 7H_2O$, 4.3; HEPES, 2.0; pH 7.4, at a concentration of 1 to 3 mg of protein per ml and were frozen and kept at −80°C before analysis. SPM-2 were diluted to 0.05 mg of protein per ml and fluorescent probes were incorporated at 35°C for 15 min with frequent vortexing. DPH was dissolved in tetrahydrofuran and TMA-DPH was dissolved in tetrahydrofuran-water (1:1). The probes were added in a volume of 0.3 to 0.5 μl/ml to give a probe concentration of 40 to 80 ng/ml. After incorporation of probe, samples were placed in the fluorimeter and maintained at 35°C. Control levels of fluorescence (base line) were determined and an aliquot of ethanol solution was added to the cuvette; fluorescence was determined 3 to 5 min later. The samples were coded and the experimenter was not aware of the source of the membranes.

Chronic ethanol treatment. Male Sprague-Dawley rats (200–300 g) were rendered ethanol-dependent by the method of Majchrowicz (1975), which entailed administering multiple doses (6–10) over a 24-hr period totaling 9 to 11 g/kg. Ethanol was administered as a 20% (w/v) solution intragastrically using a pediatric feeding tube. The animals were dosed with ethanol for 4 days, after which they were allowed to withdraw. Behavioral assessments were made hourly and the degree of intoxication or withdrawal signs were rated as described previously (Majchrowicz, 1975; Majchrowicz *et al.*, 1976).

Other methods. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the protein standard. Statistical analysis was performed using Student's *t* test for paired or unpaired samples. Multiple comparisons with a control were done by analysis of variance and Dunnett's test (Dunnett, 1964). Concentration-effect curves for membranes from control and ethanol-treated animals were compared by an analysis of variance for repeated measures.

Results

Acute ethanol administration. The effects of acute administration of ethanol on BTX-stimulated $^{22}Na^+$ uptake and the resulting blood ethanol concentrations are shown in figure 1. Ethanol was administered by intragastric intubation as a 20% (w/v) solution and the animals were sacrificed 2 hr later. At all doses studied, there was no significant effect on the uptake of $^{22}Na^+$ in the presence of BTX alone. However, at doses of 3, 4.5, and 6 g/kg there was a significant ($P < .05$) diminution in the inhibitory effect of ethanol *in vitro*. Blood

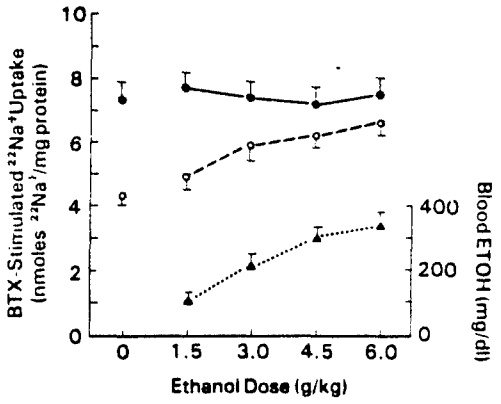


Fig. 1. Dose-response relationship for the effect of acute ethanol administration on BTX-stimulated $^{22}Na^+$ uptake. Ethanol was administered by intragastric intubation and the animals were sacrificed 2 hr later. Forebrain synaptosomes were prepared and BTX-stimulated $^{22}Na^+$ uptake was measured at 5 sec as described under "Methods" in the absence (●) or presence (○) of ethanol (400 mM) *in vitro*. The concentration of BTX was 1.5 μM. Values are the means ± S.E.M., n = 4–6 rats. Effect of ethanol *in vitro* was significantly ($P < .05$) smaller at doses of 3 g/kg and greater.

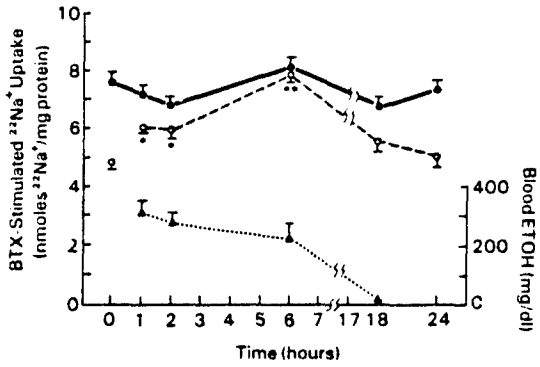


Fig. 2. Time course of effect of acute ethanol administration on BTX-stimulated $^{22}Na^+$ uptake. Ethanol (4.5 g/kg) was administered by intragastric intubation and animals were sacrificed at the indicated time. Forebrain synaptosomes were prepared and BTX-stimulated $^{22}Na^+$ uptake was measured at 5 sec as described under "Methods" in the absence (●) or presence (○) of ethanol (400 mM) *in vitro*. The concentration of BTX was 1.5 μM. Values are the means ± S.E.M., n = 4–6 rats. *$P < .05$; **$P < .01$ compared to corresponding value at time zero.

ethanol concentrations associated with the effective doses of ethanol range from 47 to 75 mM. Thus, it appeared as though the administration of a single dose of ethanol resulted in tolerance to the inhibitory effect of ethanol *in vitro* on BTX-stimulated $^{22}Na^+$ uptake. To further characterize this effect, animals were administered a single dose (4.5 g/kg) of ethanol and were sacrificed at various intervals after the dose. As shown in figure 2, as quickly as 1 hr after the dose there was a significant ($P < .05$) reduction in the inhibitory effect of ethanol *in vitro*. Six hours after the dose, when the blood ethanol concentration was 50.5 ± 8.3 mM, ethanol added *in vitro* had no effect on BTX-stimulated $^{22}Na^+$ uptake. Twenty-four hours after the dose of ethanol, the inhibitory effect of ethanol *in vitro* was similar in the control and treated groups.

In order to determine whether the observed reduction in the inhibitory effect of ethanol on BTX-stimulated $^{22}Na^+$ uptake was due to alterations in the binding of the neurotoxin, experiments were performed to measure the binding of [^{3}H]BTX-B. A single dose (4.5 g/kg) of ethanol was administered and the animals were sacrificed 6 hr later. This time point was chosen based on the results of the time course experiments (Fig. 2). The results shown in table 1 illustrate the lack of any effect of acute ethanol administration on the specific binding of [^{3}H] BTX-B in the absence or presence of ethanol *in vitro*. Similar results were obtained in experiments in which a higher concentration (75 nM) of [^{3}H]BTX-B was used (data not shown).

Chronic ethanol administration. Rats treated with repeated doses of ethanol according to the method of Majchrowicz (1975) become tolerant to, and physically dependent on, ethanol (Majchrowicz *et al.*, 1976; Kynch and Prohaska, 1981). Accordingly, animals received multiple daily doses of ethanol as described under "Methods" and were sacrificed after completing 2 days of treatment, on the day of withdrawal and at 5, 10, 20 and 35 days after withdrawal. The results from ion flux measurements are shown in figure 3. There was a significant

TABLE 1

Effect of acute ethanol administration on [^{3}H]BTX-B binding

Rats received ethanol (4.5 g/kg) or saline by intragastric intubation and were sacrificed 6 hr later. Duplicate samples of forebrain synaptosomes were incubated with [^{3}H]BTX-B (10 nM) and scorpion venom (150 μg/ml) in the absence or presence of ethanol *in vitro*. Blood ethanol concentrations were 279 ± 22 mg/dl. Values are the means ± S.E.M., n = 4–6. Values in parentheses are the percentage of inhibition of binding by ethanol *in vitro*.

Group	Ethanol	[^{3}H]BTX-B Bound
	mM	*fmol/mg protein*
Control	0	330.1 ± 16.6
Control	400	208.8 ± 10.7* (36.7%)
Acute ethanol	0	336.6 ± 16.1
Acute ethanol	400	217.6 ± 9.1* (35.4%)

* Significantly ($P < .01$) different compared to binding in the absence of ethanol *in vitro*.

TREATMENT INTERVAL	B.E.C. (mg/dl)
A. 2 days Induction	241 ± 27
B. Dependent-intoxicated	289 ± 19
C. Dependent-withdrawing	14 ± 6
D. 5 days Post-withdrawal	
E. 10 days Post-withdrawal	
F. 20 days Post-withdrawal	
G. 35 days Post-withdrawal	

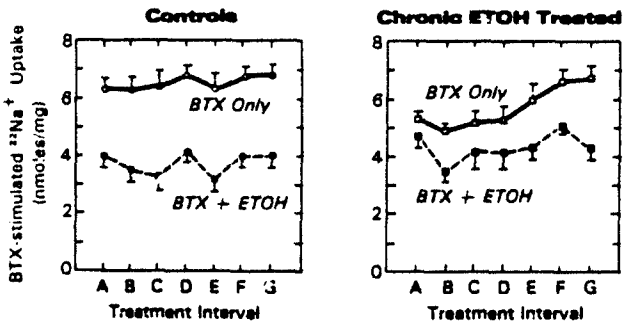


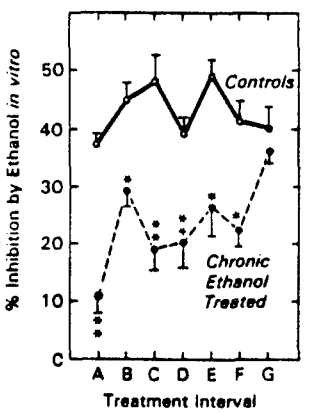
Fig. 3. Effect of chronic ethanol administration on BTX-stimulated $^{22}Na^+$ uptake. Multiple daily doses of ethanol were administered using the method of Majchrowicz (1975) and animals were sacrificed at the indicated interval. Forebrain synaptosomes were prepared and BTX-stimulated $^{22}Na^+$ uptake was measured at 5 sec as described under "Methods" in the absence (open symbols) or presence (filled symbols) of ethanol (400 mM) *in vitro*. The concentration of BTX was 1.5 μm. Values are the means ± S.E.M., n = 6–8 rats. In the chronic ethanol group, the effect of BTX only was significantly ($P < .05$ or less) smaller than corresponding control value at A, B, C and D. Also, in the chronic ethanol group, uptake in the presence of BTX and ETOH was significantly ($P < .05$) greater than the corresponding control values at E and F. B.E.C., blood ethanol concentration.

($P < .05$) decrease in the uptake of $^{22}Na^+$ in the presence of BTX alone in the ethanol-treated group after completion of 2 days of treatment, on the day of withdrawal and at 5 days after withdrawal (fig. 3, A, B, C and D). At the same time points, the addition of ethanol *in vitro* resulted in a significantly smaller degree of inhibition of BTX-stimulated $^{22}Na^+$ uptake. In the control group, the degree of inhibition by ethanol *in vitro* ranged from 36.6 ± 2.6% (fig. 3A) to 48.5 ± 4.6% (fig. 3C) whereas in the ethanol-treated group the degree of inhibition ranged from 11.2 ± 3.3% (fig. 3A) to 28.8 ± 2.0% (fig. 3B). The inhibitory effect of ethanol *in vitro* was significantly smaller in the ethanol-treated group for as long as 20 days after withdrawal (fig. 4). At 35 days after withdrawal the effect of ethanol *in vitro* was equivalent in both groups. It is of interest to note that the inhibitory effect of pentobarbital (450 μM) *in vitro* was also significantly smaller in the ethanol-treated group on the day of withdrawal and at 5 and 10 days after withdrawal (data not shown). Similar to the effect of a single dose of ethanol, chronic ethanol treatment resulted in tolerance to the inhibitory effect of ethanol *in vitro* and this tolerance was evident long after ethanol had been cleared from the body. In addition, chronic ethanol treatment also reduced the response to BTX in the absence of ethanol *in vitro*. This effect could be due to a reduced number of binding sites for BTX.

The binding of [^{3}H]BTX-B in control and ethanol-treated groups is shown in figure 5. There was no significant difference in the binding of [^{3}H]BTX-B in the absence or presence of ethanol *in vitro* at any of the treatment intervals. Similar results were obtained when a saturating concentration of [^{3}H]BTX-B (80 nM) was used (data not shown). It appears that the alterations in BTX- stimulated $^{22}Na^+$ uptake in the chronic ethanol-treated group were not due to an effect on the binding of the neurotoxin to its receptor site in the channel.

Chronic ethanol administration and membrane lipid order. The effects of chronic ethanol administration on the order or fluidity of rat brain synaptic membranes (SPM 2) were determined by measurements of the fluorescence polarization of the fluorescent probes TMA-DPH and DPH. DPH is a probe of the lower, methyl terminal portion of the lipid acyl groups (Sklar *et al.*, 1977; van Blitterswijk *et al.*, 1981) whereas TMA-DPH is a probe of the more rigid glycerol backbone regions and the carboxyl portions of the acyl groups of the membrane (Prendergast *et al.*, 1981; Harris *et al.*, 1984). The base-line fluorescence polarization of TMA-DPH and DPH was essentially identical for membranes from control and ethanol-treated rats at all times during and after the chronic ethanol treatment period (data not shown). However, chronic ethanol administration did alter the sensitivity of synaptic membranes to the disordering effects of ethanol *in vitro*. The effects of ethanol *in vitro* on the fluorescence polarization of DPH on the day of withdrawal and 5 days after withdrawal are shown in figures 6 and 7, respectively. The effect of ethanol *in vitro* on the polarization of TMA-DPH was not studied as previous studies have demonstrated that TMA-DPH is less sensitive than DPH to the effects of ethanol *in vitro* and *in vivo* (Harris *et al.*, 1984).

Chronic ethanol administration shifted the concentration-effect curve to the right, resulting in a membrane tolerance on the order of 1.5- to 2-fold. Tolerance was evident after completion of 2 days of treatment and was still present at 20 days after withdrawal. The concentration of ethanol required to decrease the polarization of DPH



TREATMENT INTERVAL	B.E.C. (mg/dl)
A. 2 days Induction	241 ± 27
B. Dependent intoxicated	289 ± 19
C. Dependent-withdrawing	14 ± 6
D. 5 days Post-withdrawal	
E. 10 days Post-withdrawal	
F. 20 days Post-withdrawal	
G. 35 days Post-withdrawal	

$^*p < 0.05; ^{**}p < 0.01$

Fig. 4. Chronic ethanol administration and percentage of inhibition of BTX-stimulated $^{22}Na^+$ uptake by ethanol (400 mM) *in vitro*. Multiple daily doses of ethanol (●) or an equivalent volume of vehicle (○) were administered using the method of Majchrowicz (1975) and animals were sacrificed at the indicated intervals. Forebrain synaptosomes were prepared and BTX-stimulated $^{22}Na^+$ uptake was measured at 5 sec as described under "Methods" in the presence of BTX (1.5 μM) and ethanol (400 mM). Values are the means ± S.E.M., n = 6–8 rats per group.

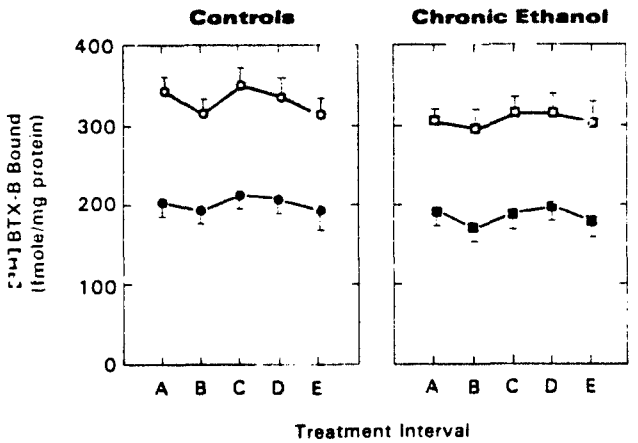


Fig. 5. Effect of chronic ethanol administration on the specific binding of [^{3}H]BTX-B. Multiple daily doses of ethanol were administered using the method of Majchrowicz (1975) and animals were sacrificed at the indicated interval (treatment interval as in fig. 8). Duplicate samples of forebrain synaptosomes were incubated with [^{3}H]BTX-B (10 nM) and scorpion venom (150 μg/ml) in the absence (open symbols) or presence (filled symbols) of ethanol (400 mM) *in vitro*. Samples were incubated at 36° for 30 min. Values are the means ± S.E.M., n = 6 per group.

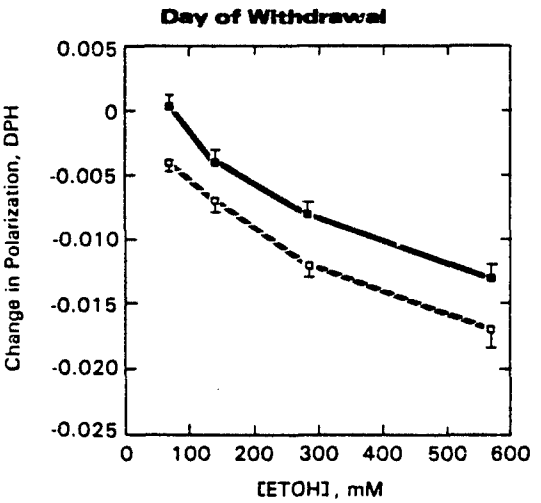


Fig. 6. Effects of ethanol *in vitro* on the fluorescence polarization of DPH. Rat forebrain synaptic plasma membrane-2 were prepared from control (□) and chronic ethanol-treated (■) animals on the day of withdrawal, approximately 6 hr after the last dose of ethanol. After DPH was incorporated, the samples were placed in the fluorimeter and maintained at 35°C. Base line levels of fluorescence were determined and an aliquot of ethanol solution was added. Fluorescence was then determined as described under "Methods." Values are the means ± S.E.M. for six membrane preparations per group. An analysis of variance for repeated measures indicated that the curves are significantly different (F = 11.30, dF = 1,10; $P < .010$).

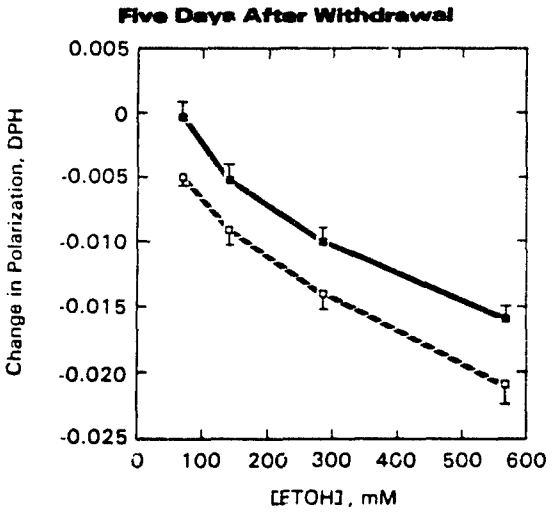


Fig. 7. Effects of ethanol *in vitro* on the fluorescence polarization of DPH 5 days after withdrawal. Rat forebrain synaptic plasma membrane-2 were prepared from control (□) and chronic ethanol-treated (■) animals 5 days after withdrawal. After DPH was incorporated, the samples were placed in the fluorimeter and maintained at 35°C. Base-line levels of fluorescence were determined and an aliquot of ethanol solution was added. Fluorescence was then determined as described under "Methods." Values are the means ± S.E.M. for six different membrane preparations per group. An analysis of variance for repeated measures indicated that the curves are significantly different ($F = 8.94$, $dF = 1,10$; $P < .025$).

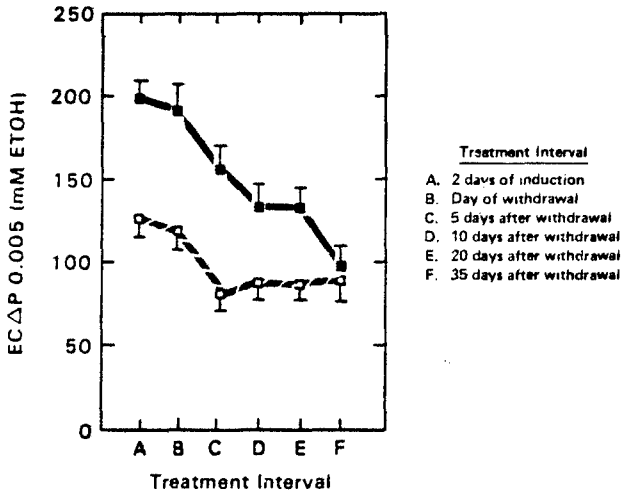


Fig. 8. Time course of altered membrane disordering by ethanol *in vitro* during and after chronic ethanol treatment. The concentration of ethanol required to decrease the fluorescence polarization of DPH by 0.005 U (ECΔ P .005) was determined by linear regression analysis of the individual concentration-effect curves at the indicated intervals during and after chronic ethanol treatment. Four concentrations of ethanol were used. Rat forebrain synaptic plasma membranes-2 from control (□) and chronic ethanol-treated (■) animals were prepared and fluorescence polarization measured as described under "Methods." Values represent the means ± S.E.M. from five to six different membrane preparations per group. Experimental group values are significantly different ($P < .05$ or less) compared to corresponding control at all intervals except F.

by 0.005 U (EC Δ P 0.005) was determined by linear regression analysis and the values obtained at each interval are shown in figure 8. The degree of tolerance to the membrane disordering effect of ethanol was estimated from the ratio of EC Δ P 0.005 ethanol-treated:EC Δ P 0.005 control. It was interesting to note that ratio of EC Δ P 0.005 values on the day of withdrawal (1.62) was remarkably similar to the values at 10 (1.48) and 20 (1.47) days after withdrawal. Thus, the tolerance produced by the chronic ethanol treatment was relatively stable and persisted for several weeks after the cessation of ethanol treatment.

Discussion

The results of the present study demonstrate that both acute and chronic ethanol treatment alter the inhibitory effect of ethanol *in vitro* on neurotoxin-stimulated $^{22}Na^+$ uptake. The effects of a single dose of ethanol were dependent on the dose administered and the time elapsed after the dose. Acute ethanol administration did not alter the action of BTX on the sodium channel in the absence of ethanol *in vitro* or the binding of [^{3}H]BTX-B, but it appeared that tolerance to the inhibitory effect of ethanol *in vitro* on BTX-stimulated sodium uptake was present. The reduced effectiveness of ethanol *in vitro* after a single dose of ethanol may be analogous to the finding of acute tolerance in whole animal studies (for review, see Cicero, 1980).

Chronic ethanol administration produced somewhat different results than the single dose studies. There was a significant reduction in BTX-stimulated sodium uptake in the absence of ethanol *in vitro* after 2 days of induction, on the day of withdrawal and at 5 days after withdrawal. Results of binding studies with [^{3}H]BTX-B in the chronic ethanol group suggest that the diminished response to BTX was probably not due to changes in the binding of BTX to its receptor site in the channel. Furthermore, the inhibitory effect of ethanol *in vitro* on sodium uptake was significantly attenuated for up to 20 days after withdrawal.

The fluidity or order of brain membranes from the chronic ethanol group was assessed by the fluorescence polarization of the probes DPH and TMA-DPH. There was no change in the fluorescence polarization of either probe at anytime during or after chronic ethanol treatment, indicating that the basal or intrinsic fluidity of brain membranes was unchanged. Intrinsic membrane fluidity has been reported to be decreased (Rottenberg *et al.*, 1981; Lyon and Goldstein, 1983; Harris *et al.*, 1984) or unchanged (Chin and Goldstein, 1977b; Beauge *et al.*, 1984) after chronic ethanol treatment. Brain membranes derived from the chronic ethanol group were resistant to the disordering effect of ethanol *in vitro* for up to 20 days after withdrawal, as measured by the change in the fluorescence polarization of DPH. Thus, adaptation to the effects of ethanol *in vitro* on sodium uptake and membrane fluidity followed a similar time course after chronic ethanol treatment.

The mechanisms responsible for the attenuated effects of ethanol *in vitro* reported here are not known. Acute *in vivo* administration of ethanol resulted in a marked attenuation of the inhibitory effect of ethanol on sodium uptake but does not appear to alter ethanol-induced disordering of reconstituted (Johnson *et al.*, 1979) or intact synaptic membranes (R. A. Harris, unpublished observation). Although it is merely speculation, these findings could be interpreted as an uncoupling of the effects of ethanol on the physical and functional properties of neurons. An analogous situation was reported recently by Mitchell *et al.*, (1985) who demonstrated that brain membranes prepared from barbiturate tolerant-dependent mice were resistant to the inhibitory effect of ethanol and pentobarbital on sodium uptake but that the disordering effects of the drugs

were unchanged. In the present study 18 to 24 hr after a single dose of ethanol the response to ethanol *in vitro* was restored to control levels suggesting that the adaptive response was transient. This uncoupling effect may be an initial but short lived component of membrane tolerance. Alternatively, it is possible that a single dose of ethanol alters the physical properties of rather specific membrane lipids that are functionally important but are undetected by existing methods used to assess the properties of bulk lipids in neuronal membranes (Taraschi and Rubin, 1985). A lipid species of this type might be present in relatively small quantities or be required in a given state of order at a critical area of the membrane in order to provide optimal conditions for efficient activity of membrane proteins.

During chronic ethanol treatment when animals are maintained in a state of prolonged intoxication, it is likely that a variety of adaptive changes occur. Indeed, after 2 days of chronic ethanol administration tolerance to most of the acute effects of ethanol has been demonstrated (Majchrowicz and Hunt, 1976; Ritzmann and Tabakoff, 1976; Goldstein and Zaechelein, 1983). At the completion of 2 days of treatment we found that brain membranes were resistant to the inhibitory effect of ethanol on sodium uptake and the disordering effect of ethanol. These data are in agreement with the data of Lyon and Goldstein (1983) which demonstrated resistance to the disordering effect of ethanol in mouse synaptic plasma membranes after 3 days of continuous exposure to ethanol vapor. Thus, like functional tolerance to the behavioral effects of ethanol, membrane tolerance to ethanol can develop rather quickly during chronic ethanol treatment.

Tolerance to ethanol in the intact animal has been reported to dissipate over a wide range of time, from a few days (Goldstein and Zaechelein, 1983; Ritzmann and Tabakoff, 1976) to weeks or months (LeBlanc *et al.*, 1969; Kalant *et al.*, 1971; Begleiter *et al.*, 1973) after withdrawal from chronic ethanol. It is apparent that the persistence or duration of tolerance in the animal is sensitive to a number of variables including the method and length of ethanol administration and the characteristics of the test used to assess tolerance. Most studies of tolerance to the membrane disordering effect of ethanol have examined a limited number of time points, usually on the day of withdrawal, although Johnson *et al.* (1980) reported that membrane tolerance was no longer evident at 12 days after withdrawal. We found evidence of membrane tolerance, assessed by the effects of ethanol *in vitro* on sodium uptake and membrane order, for up to 20 days after withdrawal. In contrast to our findings, Taraschi *et al.* (1986) reported recently that although 4 to 5 weeks of chronic ethanol treatment were required to develop tolerance to the disordering effect of ethanol in liver microsomes and erythrocytes, the tolerance was lost after 1 or 2 days of withdrawal. Similar to the findings for behavioral tolerance to ethanol it is likely that the persistence of membrane tolerance is dependent on a number of variables including the length and method of treatment, the nature of the molecular probe and the source and type of membrane preparation. In the case of the central nervous system there is a growing body of evidence that some of the functional properties of neurons are altered for prolonged periods of time after withdrawal from chronic ethanol treatment (Begleiter and Porjesz, 1977; Walker *et al.*, 1981; Eckardt *et al.*, 1986). The relationship of persistent changes in the brain to the nature of human alcoholism remains to be established.

A number of factors suggest that alterations in the functional properties of sodium channels might be involved in some of the actions of ethanol: 1) ethanol inhibits sodium influx at concentrations achieved *in vivo*, an effect that is fully reversible (Mullin and Hunt, 1984, 1985); 2) there is an excellent correlation between membrane disordering and inhibition of sodium influx (Harris and Bruno, 1985a); 3) inhibition of sodium influx by ethanol is reduced in tissue from ethanol-treated rats (this study); and 4) tolerance to inhibition of sodium influx and membrane disordering by ethanol share a common time course after chronic ethanol treatment (this study). However, further research is necessary to define in detail the involvement of neuronal ion channels in the actions of ethanol.

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